DOI: 10.7860/JCDR/2015/12062.5622

Review Article

Genetics Section

A Comprehensive Review on Clinical Applications of Comet Assay

VIDYA GUNASEKARANA[1], GLADWIN VICTOR RAJ[2], PARKASH CHAND[3]

ABSTRACT

Increased levels of DNA damage and ineffective repair mechanisms are the underlying bio-molecular events in the pathogenesis of most of the life-threatening diseases like cancer and degenerative diseases. The sources of DNA damage can be either exogenous or endogenous in origin. Imbalance between the oxidants and antioxidants resulting in increased reactive oxygen species mostly accounts for the endogenously derived attacks on DNA. Among the various methods employed in the estimation of DNA damage, alkaline comet assay is proven to be a relatively simple and versatile tool in the assessment of DNA damage and also in determining the efficacy of DNA repair mechanism. The aim of this article is to review the application of comet assay in the field of medicine towards human biomonitoring, understanding the pathogenesis of cancer and progression of chronic and degenerative diseases, prediction of tumour radio & chemosensitivity and in male infertility. A standardized protocol and analysis system of various variants of comet assay in different types of cells, across the labs will be of useful and reliable clinical tool in the field of Medicine for the estimation of levels of DNA damage and repair mechanisms.

Keywords: Bio-monitoring, Cancer, Comet assay, DNA damage, Infertility, Occupational exposure

INTRODUCTION

Human body cells are constantly exposed to harmful factors which have the potency to cause DNA damage. Most of these harmful factors are of oxidative in nature [1,2]. Based on the sources from which these harmful factors are generated, they can be broadly categorized into exogenous and endogenous in origin as enumerated in [Table/Fig-1] [2-4]. Endogenous factors originate intracellular during the normal process of metabolism and also pathologically during inflammation. Attacks from both exogenous as well as endogenous factors result in DNA damage [3-9].

Endogenous sources	Exogenous sources
Reactive oxygen species, a product of normal metabolic process.	Plant toxin
Estrogen metabolites	UV-rays, X-rays and gamma rays
Methylating agents, Reactive nitrogen species	Mutagenic chemicals like arsenic, mercury etc. that act as DNA intercalating agents
Hydrolysis of the glycosylic bond in DNA, and hydrolytic deamination	Chemotherapeutic agents and radiotherapy
Carbonyl stress (Reactive carbonyl species)	Viruses

[Table/Fig-1]: Sources of DNA damage UV Rays –Ultraviolet Rays

The dreaded effects of unrepaired DNA damage are mutations, genetic recombination, premature apoptosis, chromosomal aberrations, tumour formation, cell death and it forms the pathological basis of various disease conditions [2,9]. But there are certain innate DNA repair mechanisms which include photo-reactivation, nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) to withstand the DNA damage and to restore the genetic stability. Contrary to the ineffective DNA repair mechanisms being the hallmark in the pathogenesis of cancers, recent studies suggest that certain types of tumour cell possess increased DNA repair capacity which may cause resistance to therapeutic agents and affect the outcome of therapy [9-11].

Assessment of DNA damage and repair mechanism

The challenges of assessment methods being, it should be sensitive, rapid, simple, and able to assess damage in both proliferating as well as non-proliferating cells. There are several assessment methods available to assess the DNA damage. The merits and demerits of each of this molecular procedure have been clearly depicted in [Table/Fig-2] [9-15].

Sl. No.	Procedure	Advantages	Limitations
1.	PCR	Easy to measure gene-specific DNA damage	Cannot quantify & recognize the kind of damage [13]
2.	TUNNEL	Detects single & double strand breaks by fluorescinating the free ends of DNA	Cannot differentiate apoptosis from necrosis & autolysis [14]
3.	HPLC	Sensitive & specific for quantification of thymidine dimer & oxidative DNA damage	Early elution property of liquid chromatography
4.	Micro-nucleus Assay	Easy to carry out the procedure less time consuming	Low sensitivity
5.	Halo Assay	Detect alteration in DNA organization in individual cell	Low sensitivity
6.	FISH	Non-isotope labeling & detection method More sensitive than FCM	Well-equipped laboratory facility
7.	FCM	Detects DNA damage exclusively in apoptotic cells	Sensitivity is low compared to FISH [15]
8.	GC-MS	Sensitive to detect oxidative DNA damage	Over estimation of damage
9.	Immunological Assay	Requires less amount of DNA	Needs costly equipment
10.	Comet Assay	Detects damage in individual, non-proliferating cell	Damage due to small deletions cannot be detected
		Differentiate viable, apoptotic & necrotic cells	

[Table/Fig-2]: DNA damage detection studies
PCR - Polymerase chain reaction, TUNEL – Terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling, HPLC- High-performance liquid chromatography, FISH - Fluorescence in situ hybridization, FCM - Flow cytometry, GC-MS - Gas chromatography-mass spectrometry

Merits of Comet Assay over other molecular diagnostic methods

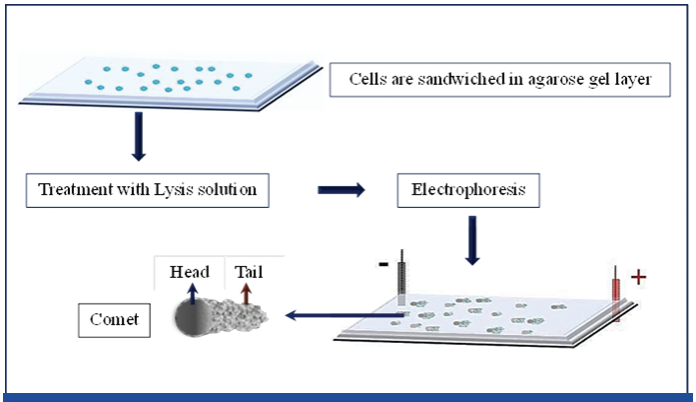
Compared to the various techniques listed, studies have shown that the Single cell Gel Electrophoresis or the Comet assay is highly sensitive method to detect low levels of DNA damage. Further the results can be obtained in a relatively short period of time even with less number of proliferating/non-proliferating cells. In addition, deployment of wide range of cells; peripheral blood mononuclear cells, buccal epithelial cells, nasal epithelial cells, lens epithelium, tear duct epithelial cells, sperms as well as biopsy tissues in comet assay makes it a versatile and potential tool of choice to assess the DNA damage and repair efficiency [16,17].

Comet assay

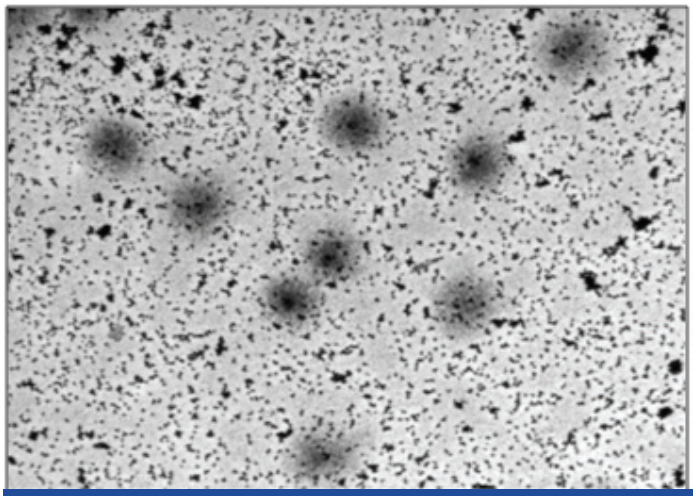
The basic principle of the Comet assay is the movement of negatively charged damaged low molecular weight DNA fragments towards anode during electrophoresis leaving a trail resembling a tail of comet [18,19]. *Treatment of cells with lysis solution which is hypertonic, and non-ionic detergent, removes the cell membrane, cytoplasm and nucleoplasm including nucleosomes.* The damaged DNA in the leftover nucleoid mass when subjected to electrophoresis migrates towards the anode producing a comet-like structure. The measurement of this tail reflects the extent of DNA damage [18-24]. The steps involved in comet assay have been depicted in the [Table/Fig-3]. The comet images of the peripheral blood lymphocytes (PBL) showing minimal, moderate and extensive DNA damage are shown in the [Table/Fig-4-6] respectively.

Factors influencing comet assay

The validity and the reliability of the comet assay are influenced by various factors. Optimum results are obtained if standardized protocol is followed. The concentration of the agarose in the gel influences the comet assay, lesser the concentration more the tail length. Though the period of lysis has minimal effect, alkaline incubation time plays a significant role [20]. The percentage of DNA in the tail increases with the duration in the alkaline solution. The parameters which have greatest influence in the comet tail length and the percentage of DNA in the tail are the electrophoresis voltage gradient, duration and the temperature. Within limits, the tail intensity and length of comet increases proportional to the voltage and the time duration of electrophoresis. It has been suggested to run the electrophoresis in a cold room so that the tank temperature is maintained below 15°C [20].



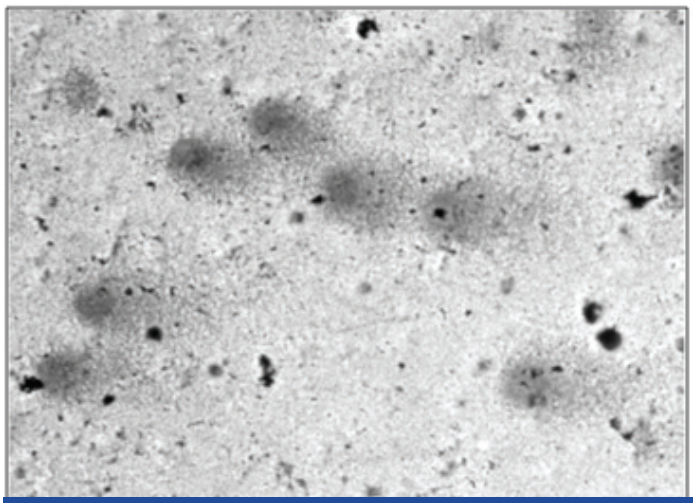
[Table/Fig-3]: Steps involved in Comet assay



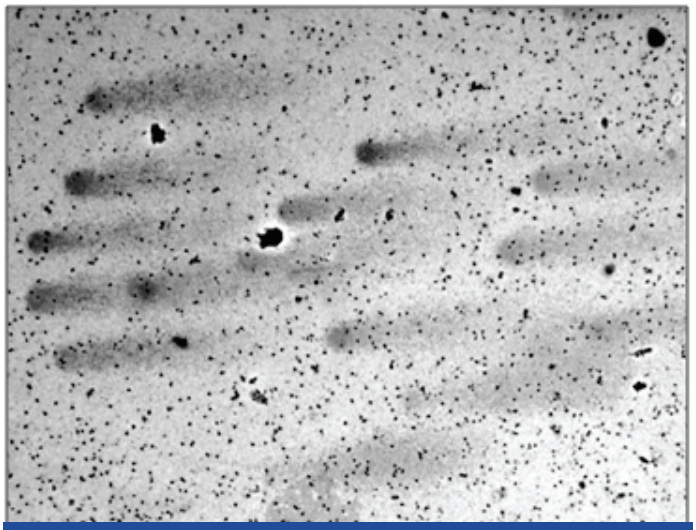
[Table/Fig-4]: PBL cells showing minimal DNA damage

Modified versions of Comet assay

Comet assay underwent several technical modifications since its introduction, thereby widening its application. The alkaline version of Comet assay was introduced in the year 1988, which is now the most commonly used and widely accepted method for the detection of single and double strand breaks [20,25]. Alok Dhawan et al., demonstrated the extent of DNA damage using comet assay in both eukaryotic and prokaryotic cells in their in vitro and in vivo studies. Further, attempts were made by Marcos et al., to augment the ability of the comet assay to determine the specific excision repair sites by including DNA repair inhibitors, synthesis inhibitors or chain terminators. Hydroxylurea and cytosine arabinoside were also been incorporated to the technique to detect the lesions produced by the alkylating agents like ethyl methane sulfonate and methyl methane sulfonate in different cell systems [17,25,26]. Comet assay with incorporation of lesion-specific endonucleases enabled detection of specific DNA damage as shown in the [Table/Fig-7] [20,27].



[Table/Fig-5]: PBL cells showing moderate DNA damage



[Table/Fig-6]: PBL cells showing extensive DNA damage

Comet assay combined with Endonucleases	Specific DNA lesions detected
Formamidopyrimidine DNA glycosylase (FPG)	Formamidopyrimidines, oxidized purines, ring-opened N7 guanine adducts produced by alkylating agents
8-oxo-guanine (8-oxoG) DNA glycosylase (OGG1)	oxidized purines and formamidopyrimidines
Endonuclease III	oxidized pyrimidines
T4 endonucleaseV	dimerized pyrimidines (induced by UV)
3-methyladenine DNA glycosylase II (AlkA)	3-methyladenine
uracil DNA glycosylase (UDG)	uracil misincorporated in DNA
Human FEN-1	Double strand break repair and base excision repair

[Table/Fig-7]: Comet assay in combination of lesion-specific endonucleases

Recent Advances in Comet Assay – its merits

Comet assay in combination with fluorescent labeled probes targeted against particular DNA sequences constitute *Comet-FISH assay*, which has been used to assess the DNA damage and repair of single genes or short DNA sequences [27]. The shift of fluorescent signals from the comet tail to the head in subsequent assays overtime can be monitored to evaluate the effectiveness of DNA repair in specific genes/sequences. With this recent development, it has been shown that Comet–FISH assay can be used as a substitute to ligation –mediated PCR and southern blotting techniques to assess the efficacy of transcription-coupled repair (TCR) mechanism in significant levels of DNA damage [27].

A modified version of comet assay-*Apo-necro Comet assay* that has been devised recently to differentiate viable, apoptotic and necrotic cells thereby determines the viability status of individual cells [17,20].

Cortes-Gutierrez et al., successfully isolated nuclear subunits -chromosomes and subjected those to electrophoresis for identification of DNA damage in isolated chromosomal mass by a technique called as *Chromosome-Comet assay* [20,28].

The limitation of processing few samples at a given time has been overcome by means of *High throughput – Comet assay* using multi chamber plates and high throughput techniques. A combination of multiple gels on one substrate, and reduction of the volume of agarose gel per sample has improved the throughput of the comet assay [20].

Application of comet Assay in the field of Medicine

The comet assay has a wide range of applications in both environmental health and medical science. It includes bio-monitoring of human populations for occupational/environmental exposure to genotoxic agents, assessment of DNA damage; protective effect of antioxidants and DNA repair capacity in various diseases, particularly in cancer [18,27]. The versatility of alkaline comet assay to detect a wide range of lesions such as single and double-strand breaks, AP sites, repair intermediates has enabled it as a tool of choice for risk assessment, early diagnosis of the disease, therapy monitoring and prognosis of diseases like cancer [19,29]. Apart from this comet assay has its role in drug toxicity monitoring, UV radiation injury, phototherapy induced DNA injury, assessment of sperm DNA damage in male infertility etc [18,29].

Biomonitoring of Environmental and Occupational Exposure

Comet Assay is a valuable tool in measuring the extent of DNA damage and its quantification in populations exposed to different types of genotoxic agents and environmental pollutants. The degree of damage in association with the dose and period of exposure to toxic agents can be measured using comet assay which helps in early detection of health risks in populations exposed to environmental toxic agents. Studies have shown that the comet assay was used to evaluate the impact of living near the source of environmental pollutants, like crude oil refineries, factories, waste incinerators etc., and along with other markers of oxidative stress, to study the oxidative potential of particulate matter (PM) exposure [30,31].

PBL cells of persons exposed to chronic low dose radiation, antineoplastic drugs, volatile organic compounds, metals etc., showed increased levels of DNA damage which correlated with the structural chromosomal aberrations, micronucleus assay and markers of oxidative damage [30,31].The advantage of comet assay over the other eco-toxicological parameters or assays being simultaneous assessment of repair kinetics and apoptotic cells with DNA strand breaks. The comet responses in detecting DNA damages have been found to be more sensitive, rapid and reproducible in comparison to the other assays [29].

Comet Assay and Cancer

In Vivo Comet – Assessment of Geno-toxicity

The International Conference on Harmonization [ICH- S2 (R1)] guidance (2012) has recognized the In vivo comet assay, in addition to In vivo micronucleus assay, as a potential tool for the assessment of genotoxic potential of chemicals/carcinogens [32]. The ability of In vivo comet assay in differentiating the genotoxic and non genotoxic chemicals has been widely utilized in the field of genotoxicology. The comet assay can be employed as a prospective biomarker in prediction of genetic susceptibility and in detection of DNA damage leading to carcinogenicity, in addition to assessment of local genotoxicity in tissues and all cell types that are otherwise not possible with other standard assessment methods [32].

Assessment of DNA damage in patients with cancer

Increased levels of DNA damage and ineffective or defective DNA repair mechanisms are the underlying molecular events driving the initiation of pathogenesis and progression of cancer. Therefore, comet assay has been extensively used in the assessment of DNA damage in PBL and tumour cells and the characteristics of various DNA repair mechanisms with a wide range of DNA damaging agents. Extensive studies were done in patients with various types of cancer which showed invariably increased in levels of basal DNA damage and defective DNA repair mechanism [33-38]. Further, attempts were made to determine the correlation between the levels of DNA damage to the various types and different stages of cancer. The levels of DNA damage thereby provide valuable information regarding the nature and severity of the cancer which could be a useful tool for the specialists treating the cancer to determine the best possible intervention and treatment modality in halting the progression and to aim for a cure in patients with cancer [33].

Prediction of tumour radio & chemosensitivity

The success and outcome of various treatment modalities in patients with cancer depends upon the nature of the tumour cell in its intrinsic ability to proliferate, status of hypoxia and to a large extent its sensitivity towards radiation and various chemotherapeutic agents. Clonogenic cell survival assay and functional imaging of tumour cells by means of PET scan and single photon emission CT has been employed to measure the response towards radiation, the metabolism and hypoxia status. The rate limiting step and the disadvantage in the clonogenic cell survival assay being the ability and the time taken by the tumour cells to grow in soft agar. The disadvantages of the above mentioned methods have been

overcome by the alkaline comet assay in measuring the sensitivity of tumour cells towards low levels of radiation that are practically deployed in the treatment of patients with cancer and also in cells with low proliferative ability [33]. Attempts were made to assess the radio-sensitivity of different tumour cells lines of colon, bladder [34,35], breast [36,37], prostate [38] cancer and have found that the alkaline comet assay to be a useful tool. Further, the efficacy of various doses of radiation and radiation in different stages of cancer has been studied. Thus a standardized and validated alkaline comet assay will be of useful tool in predicting the radiosensitivity of the tumour cells and in determining the efficacy and progress of radiotherapy treatment in patients with cancer. The advantage of measuring DNA cross linking and alkylation in addition to the assessment of breakages involving single and double strands of DNA further potentiates the alkaline comet assay to be a versatile tool in assessing the sensitivity towards various chemical compounds which are used as chemotherapeutic agents in the treatment of cancer [16,20,33]. A clear dose-response and time response relationship with DNA double strand breaks was obtained by comet assay which shows the potency of comet assay in radiation biodosimetry [39].

Comet assay in Chronic & Degenerative diseases

DNA damage of the target cells seems to be the final hallmark of most of the chronic diseases like Diabetes mellitus, Rheumatoid arthritis, etc. and also in neurodegenerative disorders like Alzheimer's and Parkinson's disease [31]. Higher levels of SSBs and oxidative stress were found in patients with Diabetes Mellitus compared to the healthy controls. Studies have proven that patients with chronic diseases showed increased susceptibility to free radical damage and decreased efficacy of DNA repair mechanism [16,31]. The resulted DNA damage in the above said diseased conditions is either due to increased oxidative stress as suggested by most of the studies or due to a direct insult to the DNA by means of various other toxic chemical compounds. Alkaline comet assay along with the measurement of levels of 8 hydroxy 2 deoxy guanosine seems to be an appropriate method in the assessment of DNA damage due to an oxidative insult [31].

Comet assay in Male infertility

Much of the focus have been shifted and attributed to Sperm DNA damage in cases of male infertility with otherwise normal sperm indices. The quality of sperm DNA has been assessed by comet assay and studies have shown increased evidence of sperm DNA damage in patients with male infertility compared to those of fertile men [40]. The sperm DNA damage is mainly due to the endogenously derived reactive oxygen species from polyunsaturated fatty acids. Comet assay is found to be a more sensitive technique in the evaluation of sperm DNA damage and fragmentation compared to conventional TUNEL, HALO assay, Flow Cytometry, etc [15,40]. The challenges in the assessment of sperm DNA being the modification and optimization of the comet assay technique due to the unique structure of DNA in sperm (protamine replacing histone) in comparison with other cells. Further, the paucity of DNA repair mechanisms has to be considered in application of assay in the assessment of sperm DNA. Further studies are in process to understand the mechanisms of paternal DNA damage as a cause for early loss of developmental stages [29].

CONCLUSION

The relative rapidity and simplicity of the comet assay, the various modified versions available, and the wide range of cells deployed in the comet assay, makes it a versatile and potential tool for estimating the extent of DNA damage and repair efficacy in various clinical conditions like carcinoma, chronic diseases like Alzheimer, Parkinson's etc., and infertility. There exist quite an ample number of reports on the confounding factors of comet assay in various studies. Hence, it is relevant to identify all the confounding factors that may affect the estimation of the basal level of DNA damage in individual cells. Thus comet assay can be used as potential tool in the management of cancer and other chronic diseases involving DNA damage, if the fundamental issues relating to experimental validation, standardization and data interpretation are optimized.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.
2. Assistant Professor, Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.
3. Professor and Head, Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Gladwin Victor Raj,
Assistant Professor, Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.
E-mail: drgladwin@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Nov 14, 2014**
Date of Peer Review: **Jan 27, 2015**
Date of Acceptance: **Feb 02, 2015**
Date of Publishing: **Mar 01, 2015**